Membrane Topography of the Coupling Ion Binding Site in Na\textsuperscript{+}-Translocating F\textsubscript{1}F\textsubscript{0} ATP Synthase

Christoph von Ballmoos\textsuperscript{±}, Yvonne Appoldt\textsuperscript{±}, Josef Brunner\textsuperscript{§}, Thierry Granier\textsuperscript{II}, Andrea Vasella\textsuperscript{II}, and Peter Dimroth\textsuperscript{*,*}

Institut für Mikrobiologie der Eidgenössischen Technischen Hochschule, ETH Zentrum, CH-8092 Zürich, Switzerland, Institut für Biochemie der Eidgenössischen Technischen Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland, Laboratorium für Organische Chemie der Eidgenössischen Technischen Hochschule, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

* To whom correspondence should be addressed. Phone: 0041 1 632 33 21. Fax: 0041 1 632 13 78. E-mail: dimroth@micro.biol.ethz.ch.

± Institut für Mikrobiologie der Eidgenössischen Technischen Hochschule.

§ Institut für Biochemie der Eidgenössischen Technischen Hochschule.

II Laboratorium für Organische Chemie der Eidgenössischen Technischen Hochschule.

Running title: Topography of the Na\textsuperscript{+}-binding site
A carbodiimide with a photoactivatable diazirine substituent was synthesized and incubated with the Na\(^+\)-translocating F\(_1\)F\(_0\) ATP synthase from both *Propionigenium modestum* and *Ilyobacter tartaricus*. This caused severe inhibition of ATP hydrolysis activity in the absence of Na\(^+\) ions but not in its presence, indicating the specific reaction with the Na\(^+\)-binding cE65 residue. Photocrosslinking was investigated with the substituted ATP synthase from both bacteria in reconstituted 1-palmitoyl-2-oleyl-\(sn\)-glycero-3-phosphocholine (POPC)-containing proteoliposomes. A subunit c/POPC conjugate was found in the illuminated samples but no a-c crosslinks were observed, not even after ATP-induced rotation of the c-ring. Our substituted diazirine moiety on cE65 was therefore in close contact with phospholipid but does not contact subunit a. Na\(^+\)\(_{\text{in}}\)/\(^{22}\)Na\(^+\)\(_{\text{out}}\) exchange activity of the ATP synthase was not affected by modifying the cE65 sites with the carbodiimide, but upon photoinduced crosslinking, this activity was abolished. Crosslinking the rotor to lipids apparently arrested rotational mobility required for moving Na\(^+\) ions back and forth across the membrane. The site of crosslinking was analyzed by digestions of the substituted POPC using phospholipases C and A\(_2\), and by mass spectroscopy. The substitutions were found exclusively at the fatty acid side chains, which indicates that cE65 is located within the core of the membrane.
F$_1$F$_0$ ATP synthases are large protein complexes within the membranes of mitochondria, chloroplasts, or bacteria that use an electrochemical H$^+$ or Na$^+$ gradient across the membrane to synthesize ATP. The F$_1$ portion harboring the catalytic sites for ATP synthesis protrudes from the membrane and has the universal subunit composition $\alpha_3\beta_3\gamma\delta\epsilon$. Its high-resolution crystal structure from bovine mitochondria (1) was in remarkable agreement with the binding change mechanism (2) suggesting a rotary catalytic mechanism, which was proven experimentally (3). The $\gamma$ and $\epsilon$ subunits form the central stalk protruding from the more compact $\alpha_3\beta_3$ cylinder and make a connection with the oligomeric c-ring of the membrane-intrinsic F$_0$-moiety (4, 5). Subunits $\gamma$, $\epsilon$, and c$_n$ could be crosslinked without loss of function (6) and were shown to represent the rotor by direct visualization of rotation with an attached actin filament (3, 7, 8).

Besides c$_n$, the membrane-bound subunit a is part of the F$_0$-motor which is thought to use the electrochemical ion gradient to generate rotary torque (9 – 11). As the structure of the a subunit is not known in any detail, its role in the ion translocation and torque-generating mechanism remains speculative. The a subunit is connected laterally with the c$_n$-ring (12 –15), where it is held in place by the two b subunits which form the peripheral stalk connecting subunit a with an $\alpha$ subunit of F$_1$ with the help of the $\delta$ subunit (16, 17).

Recent structural data have shown that the number of c subunits forming the rotor ring varies among species, being 10 for yeast mitochondria (5), 14 for spinach chloroplasts (18) and 11 for the Na$^+$-translocating ATP synthase from the bacterium Ilyobacter tartaricus (19). The c oligomer plays a profound role in the ion translocation and hence, its structure is of primary importance to understand the function of the rotary F$_0$-motor. In the Na$^+$-translocating ATP synthases from Ilyobacter tartaricus (20) and Propionigenium modestum (21), Q32, E65 and S66 of the c subunits serve as Na$^+$ binding ligands (22), while equivalents of cE65 serve as proton binding sites in H$^+$-translocating ATP synthases (23). This acidic residue has been implicated from an NMR$^1$ structure of monomeric subunit c of E. coli in an organic solvent.
mixture to be located in the core of the membrane (24). In a model derived from a secondary NMR structure of subunit c from P. modestum (25), cE65 was placed closer to the membrane surface, explaining numerous data on the direct accessibility of this Na⁺ binding residue from the aqueous environment (26 – 28).

In order to test these two hypotheses experimentally we took advantage of the fact that cE65 is specifically modified with dicyclohexylcarbodiimide (DCCD). We have chemically synthesized a carbodiimide derivative with a photoactivatable diazirine ring, modified cE65 accordingly and analyzed the crosslink products formed upon illumination in reconstituted proteoliposomes. We show here that crosslinking occurs specifically with the fatty acid side chains of the phospholipids, demonstrating the location of cE65 to be in the core of the cytoplasmic membrane.

EXPERIMENTAL PROCEDURES

Materials—Solvents and chemicals were purchased from Fluka, Switzerland. 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyliodide was prepared as described (29). Di-bis-phenylmethylcarbodiimide was a gift from Brian Beechey.

POPC was purchased from Avanti Polar Lipids (Alabaster, AL). HPLC grade chloroform was supplied by Amtech-Chemie, Switzerland. Fractogel TSK-DEAE-650 column material was purchased from Merck. Bio-Beads SM-2 (polystyrene beads) were from Biorad. Phospholipase C and phospholipase A₂ were purchased from Sigma.

General methods—Thin layer chromatography was performed on Merck silica gel 60F-254 plates. Intermediates and carbodiimides were detected by fluorescence quenching in UV-light (254 nm) or with a carbodiimide reaction spray (30). Flash chromatography was performed with Merck 60 (0.04-0.063 mm) silica gel in various solvent mixtures. UV spectra (λ_max in nm (log e)) were recorded in a 1 cm quartz cell. IR spectra were recorded from a 3% CHCl₃ solution. ¹H-
NMR (300MHz) and $^{13}$C-NMR(75MHz) were recorded on a Varian Gemini system. Chemical shifts $\delta$ are in ppm and coupling constants $J$ in Hz.

**Chemical synthesis of carbodiimides**—All the applied carbodiimides were synthesized by reaction of iminophosphoranes with isocyanates (31, 32). $N$, $N'$- Dibenzylcarbodiimide (33) and $N$-benzyl-$N'$-cyclohexylcarbodiimide (34) have already been described. The synthesis of the diazirine derivative of $N$-benzyl-$N'$-cyclohexylcarbodiimide ($4$, Diazirine-BCCD) is shown in Fig. 1 and described in detail below.

**Synthesis of 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl azide (2)**—A solution of 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzylidnium (1, 200 mg, 0.613 mmol) in 6 ml MeOH was treated with NaN$_3$ (87 mg, 1.38 mmol) and stirred for 4 h at 40°C in a water bath. The reaction mixture was allowed to cool to room temperature and the solvent was evaporated *in vacuo* at a temperature below 30°C. Flash chromatography was performed in hexane:ethylacetate (6:1). The residue was briefly dried *in vacuo* to yield 140 mg (95%) of (2).

$R_f$ (hexane)= 0.3. UV (CHCl$_3$): 247 (3.2), 270 (2.7), 353 (2.5). IR (CHCl$_3$): 2927 m, 2855 m, 2103 s, 1613 w, 1520 w, 1456 w, 1344 m, 1178 s, 1161 s, 1099 w, 1054 m, 1013 m, 1099 w, 1054 m, 1013 m, 939 s. $^1$H-NMR (CDCl$_3$): 4.36 (s, ArCH$_2$); 7.21 (d, $J= 8.1$, 2H); 7.35 (d, $J= 8.1$, 2H). $^{19}$F-NMR (CDCl$_3$): -65.0 (s, CF$_3$). $^{13}$C-NMR (CDCl$_3$): 54.15; 127.19; 128.7; 129.38; 137.5. HR-MS (EI) calcd for C$_9$H$_6$F$_3$N$_3$: [M – N$_2$]$^+$=213.0509 found 213.0509.

**Synthesis of N-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl-N'-cyclohexyl-carbodiimide (4)**—A solution of 4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl azide (2, 130 mg, 0.539 mol) in 4 ml CHCl$_3$ was mixed with triphenylphosphine (152 mg, 0.575 mmol) and cyclohexylisocyanate (3, 0.095 ml, 0.740 mmol), and stirred for 12 h at 40° C. The reaction mixture was cooled to room temperature and 4-phenyl-3H-1,2,4-triazolin-3,5-dione was added until a red color persisted, and then evaporated *in vacuo*. Flash chromatography was performed
in hexane:ethylacetate (6:1, 1% triethylamine). The residue was dried in *vacuo* to yield 70 mg (40%) of (4).

R$_f$(hexane)=0.1. UV (CH$_2$Cl$_2$): 247 (3.2), 270 (2.5), 371 (2.1). IR (CHCl$_3$): 3059 w, 2934 m, 2857 w, 2124 s, 1689 w, 1517 w, 1439 m, 1344 m, 1261 w, 1161 m, 1117 w, 1017 w. $^1$H-NMR (CDCl$_3$): 1.05-1.35 (m, 5H); 1.45-1.85 (m, 5H); 3.10-3.26 (m, NCH); 4.38 (s, ArCH$_2$N); 7.20 (d, J= 7.3, 2H); 7.37 (d, J= 8.7, 2H). $^{19}$F-NMR (CDCl$_3$): -65.1 (s, CF$_3$). $^{13}$C-NMR (CDCl$_3$): 24.42; 25.33; 50.11; 126.76; 128.03; 140.3. HR-MS (EI) calcd for C$_{16}$H$_6$F$_3$N$_3$: [M – N$_2$]$^+$=294.1339 found 294.1339.

**Purification of F$_1$F$_0$ ATP synthase from Propionigenium modestum and Ilyobacter tartaricus**–The F$_1$F$_0$ ATP synthase was purified from whole cells of *P. modestum* or *I. tartaricus* by fractionated polyethyleneglycol precipitation (20, 21). The ATP synthase was resuspended in 5mM potassium phosphate buffer, pH 8.0, and stored in liquid N$_2$.

**Labeling of cE65 from purified F$_1$F$_0$ ATP synthase with carbodiimide derivatives**–Typically, 20-30 µg purified ATP synthase in 20 µl 5 mM potassium phosphate, pH 7.0, 5 mM MgCl$_2$ was incubated with 10-50 µM carbodiimide derivative from a 20 mM stock solution in MeOH. The endogenous Na$^+$ content of the buffer was ≤4 µM. For kinetic inhibition measurements, samples were taken at various times and diluted to 1 ml of the assay mixture.

**Determination of ATP hydrolyzing activity**–ATP hydrolyzing activity was determined with the coupled enzyme assay and followed spectrophotometrically as described (21).

**Preparation of lipid vesicles**–Medium-sized, 100 nm unilamellar vesicles were made from synthetic POPC by the extrusion method (35). Typically, 20 mg of POPC in CHCl$_3$:MeOH (2:1, v:v) in a round-bottomed 10 ml flask were flushed with argon, dried as a thin film under reduced pressure in a rotary evaporator for 20 min, and dried in high vacuum for 4 h. The dried lipids were resuspended in 2 ml buffer (50 mM potassium phosphate, pH 7.0, 100 mM K$_2$SO$_4$) and subjected to seven freeze-thaw cycles in liquid N$_2$ and at 37°C with short vigorous shaking after
each cycle. In order to gain unilamellar vesicles of defined size, the suspension was passed 20 times through a 100 nm membrane using a liposome extruder (LiposoFast Pneumatic, Avestin, Canada). After this procedure, the completely clarified solution was stored at 4°C.

Reconstitution of purified $F_{1}F_{0}$ ATP synthase in POPC vesicles—For the reconstitution, the preformed liposomes were treated with Triton X-100 to favor the insertion of the ATP synthase into the vesicle membrane. Afterwards, the detergent was removed by adsorption to polystyrene beads. Typically, 2 ml of the liposome suspension (10 mg/ml) was mixed with Triton X-100 and MgCl$_2$ to yield final concentrations of 0.25% and 5 mM, respectively. After adding 400 µg purified ATP synthase (lipid:protein ratio (50:1, w:w)), the suspension was incubated for 45 min at room temperature with gentle stirring. To remove the detergent, three portions of wet polystyrene beads (50, 50, 80 mg) were added at 0 min, 45 min and 90 min. The suspension was then carefully separated from the beads and the proteoliposomes collected by ultracentrifugation (50 min, 200'000 g, 4°C). The supernatant was removed and the pellet resuspended in 400 µl of reconstitution buffer (50 mM potassium phosphate, pH 7.0, containing 100 mM K$_2$SO$_4$ and 5 mM MgCl$_2$) and stored at 4°C.

ATP-dependent $H^+$-uptake into proteoliposomes—ATP-dependent $H^+$-transport into proteoliposomes by reconstituted $P. modestum$ ATP synthase was measured as described (36). The quenching of ACMA fluorescence was monitored with a RF-5001PC spectrofluorometer (Shimadzu) using excitation and emission wavelengths of 410 nm and 480 nm, respectively.

Labeling of the $F_{1}F_{0}$ ATP synthase reconstituted in POPC vesicles with Diazirine-BCCD (4) and crosslinking—A suspension of the $F_{1}F_{0}$ ATP synthase (350 µl, 350 µg protein) was incubated with 200 µM Diazirine-BCCD (4) (20 mM stock solution in MeOH) for 30 min at room temperature to attach this molecule to Glu65 of the c-oligomer. The proteoliposomes were diluted to 2 ml with reconstitution buffer, centrifuged (50 min, 200’000 g, 4°C), and resuspended in 300 µl reconstitution buffer. A sample of 240 µl was then mounted 10 cm in front of a Hg-
lamp and irradiated for 40 s at $\lambda > 320$ nm and 280 W (350 W Hg-Lamp, SUSS LH 1000 lamp house). One portion (80 µl) was kept at 4°C as a control. A second 80 µl portion was mixed with 650 µl 100 mM potassium phosphate, pH 7.3, 21 µl Triton X-100 (20%) and 12.4 U phospholipase C and incubated at 37°C overnight. The third 80 µl portion was added to 650 µl 100 mM Tricine/KOH buffer, pH 8.9, 21 µl Triton X-100 (20%), 5 mM CaCl$_2$ and 14.5 U phospholipase A$_2$ and incubated at room temperature overnight.

$Na^+$-exchange experiments–The $Na^+$-exchange measurements were performed as described (37).

Preparation of subunit c and derivatives for MALDI analysis–Subunit c of the ATP synthase of *I. tartaricus* was extracted with organic solvents as described (38). Typically, a proteoliposome sample (80 µl, 80 µg protein) was mixed with a tenfold excess of CHCl$_3$:MeOH (1:1, v:v) to precipitate insoluble proteins. After centrifugation, 160 µl water was added to achieve phase separation. The lower organic phase containing the hydrophobic c-subunits and POPC was collected and an equal volume of MeOH was added. In order to remove the lipids, the sample was applied to a Fractogel TSK-DEAE column (1.5 cm x 0.3 cm), equilibrated with CHCl$_3$:MeOH (1:1, v:v). The column was washed with CHCl$_3$:MeOH:H$_2$O (4:4:1, v:v:v), and subunit c and derivatives were subsequently eluted with 50 mM ammonium acetate in CHCl$_3$:MeOH:H$_2$O (4:4:1, v:v:v). The sample was then evaporated to dryness in a Speed-Vac and stored at –20°C.

MALDI analysis–Molecular masses were determined on a Perseptive Biosystems Voyager Elite System, a MALDI-TOF instrument with reflector. The measurements were made in the linear positive mode to avoid decomposition of the crosslinker in the reflector mode. The instrument has an accuracy of ± 0.1% in the linear mode. Immediately prior to use, the dried samples were redissolved in CHCl$_3$:HCOOH (1:1, v:v), and placed on the target onto a layer of sinapinic acid, which was deposited before from a saturated stock solution in acetonitrile:water
(2:1, v:v), containing 0.1% trifluoroacetic acid. The samples were dried under a stream of nitrogen and directly subjected to measurement.

RESULTS

Reaction of carbodiimide derivatives with Na\textsuperscript{+}-translocating F\textsubscript{1}F\textsubscript{0} ATP synthase—Work described below was carried out initially with the Na\textsuperscript{+}-translocating F\textsubscript{1}F\textsubscript{0} ATP synthase from \textit{Propionigenium modestum}, but as the very closely related ATP synthase from \textit{Ilyobacter tartaricus} is much easier to prepare we switched later to this enzyme; in fact the c subunits from these two enzymes, which are the topic of this investigation, are identical except for four conservative amino acid exchanges.

\textit{N, N'}-Dicyclohexylcarbodiimide (DCCD) specifically modifies glutamate 65 of subunit c of the Na\textsuperscript{+}-translocating ATP synthases of \textit{P. modestum} (21) or \textit{I. tartaricus} (20). This modification inhibits ATP hydrolysis activity of the enzyme, and Na\textsuperscript{+} ions provide protection from DCCD labeling (39, 40). Hence, the Na\textsuperscript{+}-protected inhibition of the ATPase by carbodiimide derivatives provides a convenient assay to monitor their specific reaction with cE65. Treatment of ATP synthase with 100 µM DCCD showed rapid inactivation of ATP hydrolysis and effective protection from this inhibition was observed in presence of 50 mM NaCl (a,b, Table 1). \textit{N, N'}-Diphenylcarbodiimide (for structures, see Fig. 2) inhibited the enzyme at 200 µM but this inhibition was not protected by Na\textsuperscript{+}, indicating that cE65 was not a specific target for this compound. With 20 µM \textit{N, N'}-di-bis-phenylmethylcarbodiimide, ATP hydrolysis was inhibited in a Na\textsuperscript{+}-protectable manner indicating its reaction with cE65. It appears therefore that the substituents on the carbodiimide nitrogens must be flexible enough to permit a reaction with cE65 (Table 1).

Based on these observations, a number of carbodiimide derivatives, including one with a photoactivatable diazirine ring were synthesized and assayed for specific interaction with cE65. As summarized in Table 1, the \textit{N}-benzyl-\textit{N'}-cyclohexylcarbodiimide derived Diazirine-BCCD
(4) inhibited the ATPase in a Na\(^+\)-protectable manner, similarly as \(N, N'\)-dibenzylcarbodiimide and \(N\)-benzyl-\(N'\)-cyclohexylcarbodiimide, indicating that these compounds react specifically with cE65. All the following crosslinking experiments were accomplished with Diazirine-BCCD (4).

**Crosslink formation between substituted cE65 with subunits a or c**—Subunit c substituted with a carbodiimide is expected to rotate by ATP hydrolysis until this is blocked by steric interferences with the a subunit. Hence, if a diazirine substituent on cE65 came into close contact with subunit a, an a-c crosslink product should be formed upon illumination. This was first analyzed with the modified ATP synthase in Triton X-100 micelles by SDS-PAGE and Western blotting with polyclonal antibodies against subunits a or c. The main crosslinking products that specifically formed upon illumination were a c-multimer, probably a dimer, and an a-c adduct. However, formation of the a-c adduct was independent of ATP addition. This situation did not change by varying the concentration of the crosslinker, the incubation time, or the temperature during incubation or illumination, which indicates that the diazirine substituent on cE65 does not form close intramolecular contacts with subunit a, following ATP-driven rotation of the c-ring (data not shown). We attribute the a-c products to temporary intermolecular interactions between a and c subunits of two different ATP synthases. This conclusion was corroborated by the lack of any a-c crosslink formation if the same experiments were performed with ATP synthase reconstituted into proteoliposomes (see below).

**Reconstitution of the ATP synthase from *P. modestum* into liposomes prepared from synthetic phospholipids**—To investigate crosslinking between the substituted subunit c and phospholipids it was advantageous to use phospholipids with defined chemical structures. The F\(_1\)F\(_0\) ATP synthase was therefore reconstituted into proteoliposomes consisting of POPC. Retention of enzyme function during reconstitution was shown by measuring ATP hydrolysis (data not shown) and proton pumping activities (Fig. 3).
The ATPase activity of reconstituted proteoliposomes was severely inhibited after modifying part of the cE65 sites with the photoactivatable Diazirine-BCCD (4), quite similar to the inhibition of the detergent-solubilized enzyme (Table 1). The modification was without effect on the \( \text{Na}^{+}_{\text{in}}/\text{Na}^{+}_{\text{out}} \) exchange activity in accordance with previous results with the DCCD-modified enzyme (26). After irradiation of the sample, however, the sodium exchange activity decreased significantly to levels that are typically found in control liposomes without enzyme (Fig. 4). The \( \text{Na}^{+}_{\text{in}}/\text{Na}^{+}_{\text{out}} \) exchange activity has been attributed to the “idling” mode of the \( \text{F}_{0} \) motor performing back and forth rotations of the rotor versus the stator within a narrow angle and thereby moving \( \text{Na}^{+} \) ions back and forth across the membrane (26). This explanation is corroborated by our present data: crosslinking a modified c subunit with phospholipid (see below) abolishes the rotational motions of the rotor versus the stator that are obligatory for the exchange between internal and external \( \text{Na}^{+} \) ions.

**Identification of crosslinked products with the modified subunit c**—Crosslinked products were identified after removal of excess phospholipids by SDS-PAGE and Western blotting using polyclonal antibody against subunit c. Most of the subunit c-containing material moved as a broad zone in the area of the monomer (6.5 kDa). A second distinct band of lower intensity was seen with the mobility expected for the dimer. No other crosslinking products containing subunit c became visible, notably none with the mobility expected for an a-c product (~32 kDa). This was independent from the addition of either ADP or ATP to the reaction mixtures (data not shown). Hence, a-c crosslinks are not formed with ATP synthase reconstituted into proteoliposomes and inhibition of \( \text{Na}^{+}_{\text{in}}/\text{Na}^{+}_{\text{out}} \) exchange after photo-induction must be due to crosslink formation of subunit c with another nearby molecule, most probably a phospholipid. This was investigated by SDS-PAGE using 16.5% acrylamide for better separation of small size proteins. Very similar results were obtained with the ATPase from *P. modestum* or *I. tartaricus*, and the *P. modestum* data are exemplarily shown in Fig. 5. In the sample treated with Diazirine-
BCCD (4), a band moving somewhat slower than subunit c was found in addition to the subunit c band. After illumination, a third band of a c subunit derivative with even lower mobility became apparent indicating the formation of a crosslinked product. Its size is too small for a conjugate with another subunit, but compatible with the one for a conjugate with a phospholipid molecule.

**MALDI analysis of crosslink products**—To identify crosslink products containing subunit c, mass spectroscopic analysis was applied. These experiments were performed with the ATP synthase of *I. tartaricus*, which is easier to purify but closely related to the *P. modestum* enzyme (see above). The ATP synthase was reconstituted into POPC-containing liposomes and modified at cE65 with the Diazirine-BCCD (4). Samples were either untreated or irradiated with UV light. The modified c subunits were then isolated by extraction with chloroform/methanol (1:1, v:v), separated from excess phospholipids by ion exchange chromatography in organic solvents, and analyzed by MALDI. The results of Fig. 6 show mass spectra of modified c subunits without (A) and after irradiation (B); and assignments of the masses are given in Table 2. Subunit c modified with Diazirine-BCCD (4) has a calculated mass of 9119 Da and a corresponding peak at m/z = 9120 is clearly observable in the non-irradiated sample. Other peaks that could be assigned in this sample are the unmodified c subunit (m/z = 8797), the modified c subunit linked to the sinapinic acid matrix (m/z = 9317), and putative degradation products arising during mass spectroscopy (m/z = 9092; m/z = 9008). This phenomenon can be assured by measurements in the reflector mode, where a complete degradation of the subunit c/crosslink occurs, showing a product arising from a fragmentation of the crosslinker attached to subunit c (data not shown).

In the irradiated sample, the main peak is of m/z = 9851, corresponding to the mass calculated for subunit c modified with Diazirine-BCCD (4) crosslinked with POPC. Upon irradiation, the diazirine group was apparently completely decomposed because no peak with m/z = 9119 corresponding to subunit c with attached Diazirine-BCCD (4) was found and instead the peak with m/z = 9133 could indicate photoinduced addition products with H$_2$O and/or formic
acid. The small peak at m/z = 9731 is likely to arise by degradation of the subunit c/POPC conjugate during MALDI. These results thus indicate that in the membrane-bound ATP synthase the cE65 residues within the c subunit ring which are accessible to the Diazirine-BCCD (4) are exposed towards the phospholipid environment.

Crosslink formation of the modified subunit c with POPC could involve either the fatty acid side chains, the glycerol moiety or phosphocholine depending on the membrane topography of cE65. The approach used to discriminate between these possibilities is illustrated in Fig. 7. For this purpose, the crosslinked product was digested with either phospholipase C or phospholipase A2. Then, the modified c subunits were isolated as usual and subjected to MALDI analysis. The results shown in Fig. 6 C and Table 2 C indicate that digestion with phospholipase C converted the crosslinked subunit c/POPC product (m/z = 9851) into a new product with m/z = 9691, corresponding to the mass expected for a crosslink product with the diacylglycerol moiety of POPC. A peak at the mass of a crosslink product with the phosphocholine group (m/z = 9275) was not found, however.

The mass spectrum after digestion of the crosslinked subunit c/POPC product with phospholipase A2 was also analyzed. Two new peaks (m/z = 9584 and m/z = 9369) appeared (Fig. 6 D, Table 2 D), which match the mass of the modified c subunit bound to POPC lacking one fatty acid (oleic acid) and the mass of the modified c subunit bound to the free fatty acid (oleic acid), respectively.

These results show clearly that the crosslinking occurs exclusively with the fatty acid side chains of the phospholipids and hence that cE65 is localized within the fatty acid-containing core of the membrane (Fig. 8 A).

DISCUSSION

Considerable data accumulated over the years that the F0 subunits a and c together make up the membrane embedded rotary motor of the ATP synthase with the c subunit rotating versus
Evidence is also available that this rotation and the translocation of the coupling ions across the membrane are intimately associated events so that one cannot occur without the other. Essential insights into this model have been derived from the Na\(^+\) translocating F\(_1\)F\(_0\) ATP synthase from *Propionigenium modestum*, which provides unique experimental approaches to follow the translocation of the coupling ions across the cytoplasmic membrane. Each c subunit of the *P. modestum* ATP synthase carries a Na\(^+\) binding site with ligands contributed by Q32, E65, and S66 (22). This site becomes transiently occupied with Na\(^+\) during its translocation across the membrane. Evidence indicates a Na\(^+\)-selective subunit a channel, which connects the adjacent rotor site of c\(_{11}\) with the periplasmic surface, whereas rotor sites not in contact with subunit a have direct access from the cytoplasmic compartment (one channel model, 10). This view is consistent with the ATP-dependent occlusion of 1 Na\(^+\) per ATP synthase with a Na\(^+\)-impermeable a subunit channel (26,27,42) and it is also consistent with the catalysis of \(\text{Na}^{+\text{in}}/\text{Na}^{+\text{out}}\) exchange by F\(_1\)F\(_0\) of *P. modestum* (26). As has been pointed out previously (44), these observations are not compatible, however, with models proposing two half channels in subunit a (two channel model, 11) through which the rotor sites communicate with the two different sites of the membrane. Another important difference is that the model for torque generation by the F\(_0\) motor proposed in (10) is the only one which takes the essential role of the membrane potential into account (26, 28).

Here, we synthesized a photoactivatable carbodiimide (Diazirine-BCCD, 4), which reacted specifically with cE65. Partial modification of the rotor sites with this compound blocked ATP hydrolysis and Na\(^+\) pumping but not \(\text{Na}^{+\text{in}}/\text{Na}^{+\text{out}}\) exchange, as expected. Upon illumination, however, the exchange activity was abolished which suggests that the rotor becomes immobilized through crosslinking to phospholipids. These proved to be the targets of the photochemical reaction and hence, the cE65 site which carries the photocrosslinker must be
exposed towards the phospholipids. This is in accord with models in which the C-terminal helices of the c-subunits are on the outside of the ring (5, 43).

We clearly demonstrate that the crosslinker reacted exclusively with the fatty acid side chains of the phospholipids. This indicates that the topography of cE65 is within the core of the membrane. The length of the attached crosslinker is about 8 Å when fully extended and therefore the distance of cE65 to the glycerol moiety of POPC must be at least in the same range to explain the absence of crosslink formation with this portion of the molecule (Fig. 8 A). This result is astonishing given the overwhelming evidence for direct access of cE65 from the aqueous compartment by Na⁺ (44). It is in good agreement, however, with an E. coli model of the topography of subunit c within the membrane (43), which is compatible with the structure of the c oligomer from yeast (5).

The location of the c subunit binding sites within the membrane core is also compatible with unpublished structural data on the c₁₁₁ oligomer from I. tartaricus. In this structure, a tightly associated inner ring comprising the N-terminal helices is surrounded by an outer ring comprising the C-terminal helices. The outer helices are positioned within the grooves formed by the inner ring of helices leaving enough space between them to form access channels for the coupling ions to reach the membrane buried cE65 residue from the aqueous compartment. Hence, agreement has now been reached on the position of cE65 near the center of the membrane. This location however, by no means, decides in favor of the two-channel model. Our evidence for the direct accessibility of cE65 in case of the Na⁺ translocating ATP synthase (see above) rather warrants a modification of the one-channel model as shown in Fig. 8 B. Based on the findings presented here and elsewhere we propose that Na⁺ ions enter the a subunit channel from the periplasmic reservoir and pass through it onto the adjacent rotor site which is near the center of the membrane. From this position, the ion passes towards the cytoplasmic surface through a channel formed between an inner and two outer helices of the c₁₁₁ ring after the rotor
has turned the site out of the interface with the a channel. According to this new concept, one may want to term our model the 1a+11c-channel-model rather than the one-channel-model.

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1 The abbreviations used are: NMR, nuclear magnetic resonance; DCCD, 
dicyclohexylcarbodiimide; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; HPLC, 
high-pressure liquid chromatography; Diazirine-BCCD, N-4-[3-(trifluoromethyl)-3H-diazirin-3-
yl]benzyl-\(N^\prime\)-cyclohexyl-carbodiimide; DEAE, diethylaminoethyl; ACMA, 9-amino-6-chloro-2-methoxyacridine; MALDI-TOF-MS, matrix assisted laser desorption and ionization time of flight mass spectroscopy; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

**Fig. 1:** Synthesis of Diazirine-BCCD (4). Synthesis of \(N-4-[3\text{-}(\text{trifluoromethyl})\text{-}3H\text{-diazirin}-3\text{-yl}]\text{benzyl-}\(N^\prime\)-cyclohexyl-carbodiimide (4) from \(4\text{-}[3\text{-}(\text{trifluoromethyl})\text{-}3H\text{-diazirin}-3\text{-yl}]\text{benzyl iodide (1)}\) via \(4\text{-}[3\text{-}(\text{trifluoromethyl})\text{-}3H\text{-diazirin}-3\text{-yl}]\text{benzyl azide (2). i) NaN}_3, \text{MeOH, 4h, 40°C; ii) PPh}_3, \text{cyclohexylisocyanate (3), CHCl}_3, 16h, 40°C; 4\text{-phenyl-}3H\text{-1,2,4-triazolin-3,5-dione, RT.}

**Fig. 2:** Structures of DCCD and carbodiimide derivatives used in this study. (a) \(N, N^\prime\)-dicyclohexylcarbodiimide; (b) \(N, N^\prime\)-diphenylcarbodiimide; (c) \(N, N^\prime\)-bis-dimethylphenylcarbodiimide; (d) \(N, N^\prime\)-dibenzylcarbodiimide; (e) \(N\text{-benzyl-}N^\prime\)-cyclohexylcarbodiimide; (f) \(N-4-[3\text{-}(\text{trifluoromethyl})\text{-}3H\text{-diazirine-3-yl}]\text{benzyl-}\(N^\prime\)-cyclohexylcarbodiimide (4).

**Fig. 3:** ATP-dependent ACMA fluorescence quenching of POPC-liposomes containing *P. modestum* ATP synthase. 10-20 µl (1-2 mg lipid) of reconstituted proteoliposomes in 50 mM potassium phosphate, pH 7.0, 5 mM MgCl\(_2\) and 100 mM K\(_2\)SO\(_4\) were diluted into 1.5 ml 5 mM potassium phosphate, pH 7.0, 5 mM MgCl\(_2\), 100 mM K\(_2\)SO\(_4\) and 2 µM valinomycin was added. The quenching of fluorescence was initiated by adding 2.5 mM K-ATP and abolished with 2 µM CCCP. Fluorescence was measured using excitation and emission wavelengths of 410 and 480 nm, respectively.

**Fig. 4:** \(^{22}\text{Na}^+\text{in/Na}^+\text{out}-exchange by reconstituted *P. modestum* \(F_1F_0\)-ATP synthase in POPC-liposomes. 50 µl \(F_1F_0\)-liposomes (4 mg lipid) in 50 mM potassium phosphate, pH 7.0, 100 mM K\(_2\)SO\(_4\), 5 mM MgCl\(_2\), 100 mM NaCl were diluted into 1 ml 2 mM Tricine/KOH, pH 7.4
buffer containing 5 mM MgCl$_2$, 100 mM K$_2$SO$_4$, 100 mM choline chloride and 1 µCi carrier-free $^{22}$NaCl to generate a $\Delta p$Na$^+$ of $\sim$90 mV. The mixture was incubated at room temperature and samples were taken at the indicated times. After separating external $^{22}$Na$^+$ by cation exchange chromatography, internal $^{22}$Na$^+$ was analyzed by $\gamma$-counting. $^{22}$Na$^+$-uptake after treatment of the F$_1$F$_0$-liposomes with 100 µM cross-linker (4) for 30 min (■); $^{22}$Na$^+$-uptake after illumination (30 sec) of the cross-linker (4) treated F$_1$F$_0$-liposomes (▲).

**FIG. 5:** Separation of c subunit derivatives by SDS-PAGE. The *P. modestum* ATP synthase was reconstituted into POPC liposomes and modified with 250 µM cross linker (4). Samples containing 25 µg protein were then applied to SDS gel electrophoresis on a 16.5 % gel (45). Lane 1 shows the sample prior to illumination and lane 2 shows the sample after 40 s illumination with UV light ($\lambda$>320nm at 280 W). Arrows indicate the different subunit c products. M: protein marker (sizes are given in kDa).

**FIG. 6:** MALDI analysis of subunit c modified at E65 with Diazirine-BCCD (4) and of crosslink products. (A) The c subunit of the F$_1$F$_0$ ATP synthase from *Ilyobacter tartaricus* reconstituted into POPC liposomes were specifically modified at E65 by 30 min incubation with 200 µM Diazirine-BCCD (4) at 25°C. Excess crosslinker was then removed by ultracentrifugation and resolubilization. (B) like (A), but with subsequent irradiation ($\lambda$>320 nm) for 40 sec; (C) like (B), but with subsequent digestion at 37°C overnight with phospholipase C; (D) like (B), but with subsequent digestion at 25°C overnight with phospholipase A$_2$. The modified c subunits and crosslink products were isolated by extracting the samples with 10 volumes CHCl$_3$:MeOH (1:1, v:v) and subsequent phase separation by adding 2 volumes of water. The lower organic phase was subjected to weak anion exchange chromatography to separate the c subunit derivatives from excess phospholipids. The fractions with the c subunit derivatives were dried in a Speed-Vac. Immediately prior to MALDI analysis, samples were resolubilized in CHCl$_3$:MeOH (1:1, v:v).
Fig. 7: Model for crosslink formation between Diazirine-BCCD (4) bound to E65 of subunit c and phospholipids. Upon irradiation with UV light the diazirine group is converted into a highly reactive carbene, which immediately reacts with any neighbouring molecule. To illustrate this reaction, crosslink formation with the fatty acid portion of POPC is shown. Subsequent digestion with phospholipase C or phospholipase A2 would yield crosslink products of molecular mass m/z = 9685 or 9587 and 9373, respectively. Note that crosslink formation with the glycerol moiety or phosphocholine would yield different products with different masses after digestion with the phospholipases.

Fig. 8: A.) Approximate topography of glutamate 65 of subunit c in the lipid bilayer. The left part shows a POPC molecule with approximate dimensions and the right part shows glutamate 65 with the dimension of the attached carbodiimide crosslinker within the outer helix of subunit c. According to our results, the crosslinker reacts exclusively with the fatty acid side chains of POPC. As indicated by the yellow circle, E65 should thus be located at the level of ≥ C8 of the fatty acid side chains within the membrane.

B.) Schematic diagram on structural features of the F0 part of the Na+-ATP synthase and the path of Na+ ions through the membrane. The rotor consists of 11 c subunits and the stator consists of subunit a which is laterally connected to the rotor. The eleven Na+ binding sites on the rotor are formed with ligands from Q32 on the inner helix and E65 and S66 on outer helices. These sites are located within the core of the membrane. Subunit a contains a Na+ access channel which connects the periplasm with the c subunit binding site at the a/c interface. All other sites of the c ring are in contact through access channels inherent in c11 with the cytoplasm.

In ATP synthesis, Na+ ions following their electrochemical potential pass through the subunit a channel onto an empty rotor site at the a/c interface. Through the potential, rotation is biased to the right. After exiting the rotor/stator interface, the Na+ ion dissociates through its individual rotor channel into the cytoplasm.
### TABLE I:
Inhibition of the ATPase activity of *P. modestum* by different carbodiimide derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>c (µM)</th>
<th>% ATPase activity after 2 min</th>
<th>- NaCl</th>
<th>+ NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N</em>, <em>N'</em>-Dicyclohexylcarbodiimide</td>
<td>100</td>
<td></td>
<td>12</td>
<td>75</td>
</tr>
<tr>
<td><em>N</em>, <em>N'</em>-Diphenylcarbodiimide</td>
<td>200</td>
<td></td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td><em>N</em>, <em>N'</em>-Bis-dimethylphenylcarbodiimide</td>
<td>20</td>
<td></td>
<td>20</td>
<td>95</td>
</tr>
<tr>
<td><em>N</em>, <em>N'</em>-Dibenzylcarbodiimide</td>
<td>50</td>
<td></td>
<td>22</td>
<td>96</td>
</tr>
<tr>
<td><em>N</em>-Benzyl-<em>N'</em>-cyclohexylcarbodiimide</td>
<td>50</td>
<td></td>
<td>11</td>
<td>75</td>
</tr>
<tr>
<td><em>N</em>-4-[3-(Trifluoromethyl)-3H-diazirine-3-yl]benzyl-<em>N'</em>-cyclohexylcarbodiimide (4)</td>
<td>50</td>
<td></td>
<td>6</td>
<td>77</td>
</tr>
</tbody>
</table>

Incubation mixtures contained 30 µg protein in 5 mM potassium phosphate, pH 7.0, 5 mM MgCl₂ and 20-200 µM carbodiimide derivative with or without 50 mM NaCl.
<table>
<thead>
<tr>
<th>Identified product</th>
<th>Calculated mass</th>
<th>Mass found in MALDI (m/z), +/- 10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c subunit, unlabeled</td>
<td>8795</td>
<td>8797</td>
</tr>
<tr>
<td>c subunit, labeled with Diazirine-BCCD (4)</td>
<td>9119</td>
<td>9120</td>
</tr>
<tr>
<td>c subunit, labeled with (4), N2-cleavage during MALDI</td>
<td>9091</td>
<td>9092</td>
</tr>
<tr>
<td>c subunit, labeled with (4), partial label cleavage during MALDI (- 115 m/z)</td>
<td>9004</td>
<td>9009</td>
</tr>
<tr>
<td>c subunit, labeled with (4), sinapinic acid (Matrix-Peak)</td>
<td>9315</td>
<td>9317</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c subunit, labeled with (4), crosslinked to water, reaction with formic acid</td>
<td>9137</td>
<td>9133</td>
</tr>
<tr>
<td>c subunit, labeled with (4), crosslinked with POPC</td>
<td>9851</td>
<td>9851</td>
</tr>
<tr>
<td>c subunit, labeled with (4), crosslinked with POPC, partial label cleavage during MALDI (- 115 m/z)</td>
<td>9736</td>
<td>9731</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c subunit, labeled with (4), crosslinked with POPC, PLC digest</td>
<td>9685 (diacyl-glycerol)</td>
<td>9691</td>
</tr>
<tr>
<td></td>
<td>9275 (phospho-choline)</td>
<td>none</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c subunit, labeled with (4), crosslinked with POPC, PLA2 digest</td>
<td>9587 (minus oleic acid)</td>
<td>9584</td>
</tr>
<tr>
<td></td>
<td>9373 (oleic acid only)</td>
<td>9369</td>
</tr>
</tbody>
</table>

Abbreviations: Diazirine-BCCD (4), N-4-[3-(trifluoromethyl)-3H-diazirin-3-yl]benzyl-N’-cyclohexyl-carbodiimide; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; PLC, phospholipase C; PLA2, phospholipase A2
**Figure 1:**

![Chemical reaction diagram](attachment:image1.png)

**Figure 2:**

![Structural diagrams](attachment:image2.png)
Figure 3:

![Graph showing relative fluorescence over time (s).](image)

Figure 4:

![Graph showing time (min) vs. Na\(^+\) uptake (nmol/mg lipid).](image)
**Figure 5:**

![Image of a gel with bands labeled M, 1, and 2, and lines indicating c-subunit + CL + POPC, c-subunit + CL, and c-subunit.](attachment:image.png)

**Figure 6:**

![Image of mass spectra with peaks labeled at various mass-to-charge ratios (m/z) and corresponding intensity values.](attachment:image.png)
**Figure 7:**

![Diagram showing the biochemical interactions involving c-subunit and diazirin-BCCD.](image)

**Figure 8:**

**A**

![Diagram illustrating the structure of a subunit and the range of the crosslinker.](image)

**B**

![Diagram showing the location of Glu65 in the context of cytoplasmic and periplasmic environments.](image)
Membrane topography of the coupling ion binding site in Na+-translocating F1F0 ATP synthase
Christoph von Ballmoos, Yvonne Appoldt, Josef Brunner, Thierry Granier, Andrea Vasella and Peter Dimroth

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